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RESEARCH

Diallel Analysis of Aflatoxin Accumulation in Maize

W. Paul Williams,* Gary L. Windham, and Paul M. Buckley

ABSTRACT

Aflatoxin, a toxin produced by the fungus *Aspergillus flavus* Link:Fries, occurs naturally in maize (*Zea mays* L.). It is a potent carcinogen, and its presence markedly reduces the value of grain. Host-plant resistance to *A. flavus* infection and subsequent aflatoxin accumulation is generally considered a desirable means of reducing losses to aflatoxin. Maize germplasm lines with resistance to aflatoxin contamination have been developed in Mississippi. Four of the aflatoxin-resistant lines and six other lines were used as parents to produce a diallel cross. The diallel cross was evaluated for resistance to aflatoxin contamination in field trials conducted in Mississippi in 2005 and 2006. General combining ability (GCA) and specific combining ability (SCA) were highly significant sources of variation each year. Reciprocal effects were not significant in 2005 or in the combined analysis over years. In the analysis over years, GCA effects for reduced aflatoxin were highly significant for the four lines developed as sources of resistance: Mp313E, Mp494, Mp715, and Mp717. The GCA effect for reduced aflatoxin was also highly significant for Mo18W and NC408. These lines should be useful in developing maize lines and hybrids with resistance to aflatoxin contamination. Breeding methods that maximize the use of GCA should be effective in enhancing resistance to aflatoxin accumulation when using these germplasm lines.

USDA-ARS, Corn Host Plant Resistance Research Unit, Box 9555, Mississippi State, MS 39762. Joint contribution of USDA-ARS and the Mississippi Agric. and Forestry Exp. Stn. Journal no. J-11130. Received 26 July 2007. *Corresponding author (Paul.Williams@ars.usda.gov).

Abbreviations: GCA, general combining ability; LSD, least significant difference; NRRL, Northern Regional Research Laboratory; QTL, quantitative trait locus; SCA, specific combining ability.

Aflatoxin, produced by the fungus *Aspergillus flavus* Link: Fries, occurs naturally in maize (*Zea mays* L.). This toxin is the most potent carcinogen found in nature (Castegnaro and McGregor, 1998). Consumption of aflatoxin contaminated foods is a major cause of hepatocellular carcinoma, the fifth most common cancer worldwide (Wild and Hall, 2000).

In the United States, aflatoxin contamination of preharvest maize is a sporadic problem in the Midwest but a chronic problem in the Southeast (Payne, 1992; Widstrom, 1996). Drought and high temperatures have frequently been linked with high levels of aflatoxin contamination. The U.S. Food and Drug Administration has set a tolerance of 20 ng g^{-1} for aflatoxin in B_1, the most commonly found form of aflatoxin in maize grain. Grain with higher levels is restricted from interstate commerce (Gourma and Bullerman, 1995).

Host-plant resistance to *A. flavus* infection and subsequent aflatoxin accumulation is a highly desirable means of reducing losses to aflatoxin. Scott and Zummo (1988), working in Mississippi, identified maize germplasm that exhibited resistance to kernel infection by *A. flavus*. They developed and released Mp313E and Mp420, the first maize germplasm lines released as sources of resistance to *A. flavus* infection and aflatoxin accumulation (Scott

Published in Crop Sci. 48:134–138 (2008).
doi: 10.2135/cropsci2007.05.0306
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and Zummo, 1990b, 1992). Two additional germplasm lines developed in Mississippi, Mp715 and Mp717, have also been released (Williams and Windham, 2001, 2006). Maize germplasm with resistance to *A. flavus* infection and aflatoxin accumulation has also been identified at locations in Texas, Illinois, and Georgia (Betrán et al., 2002; Campbell and White, 1995a; McMillian et al., 1993).

In general, the agronomic quality of germplasm lines developed and released as sources of resistance to aflatoxin contamination has been poor. To use this germplasm to develop elite, aflatoxin-resistant lines suitable for producing commercial hybrids requires an understanding of the inheritance of resistance and combining abilities of the lines. Investigations into the inheritance of resistance to *A. flavus* infection and aflatoxin accumulation have been conducted at several locations.

Working in Illinois, Campbell and White (1995b) found that in general Aspergillus ear rot and aflatoxin accumulation were not highly correlated but that inbred line LB31 exhibited consistently high levels of resistance to both. In a generation means analysis of B73 × LB31, Campbell et al. (1997) showed that both additive and dominance types of gene action were important for resistance to aflatoxin production.

From diallel analyses of yellow and white inbred lines conducted at three locations in Texas, Betrán et al. (2002) found that Tx772 and FR2128, among the yellow inbred lines, expressed the highest levels of general combining ability (GCA) for reduced aflatoxin contamination across locations or at specific locations. The hybrid Mp715 × Tx772 exhibited consistently low levels of contamination across locations. For the white inbred lines, GCA for reduced aflatoxin contamination was less consistent across locations, but CML176, CML269, and CML322 exhibited significant GCA for reduced aflatoxin contamination at some locations. The investigators concluded that differences in levels of insect damage among locations contributed to differences in aflatoxin accumulation.

Aflatoxin accumulation after inoculation with *A. flavus* and infestation with southwestern corn borer, *Diatraea grandiosella* Dyar, was investigated in diallel crosses evaluated in Mississippi. Both Mp313E and Mp717, germplasm lines developed and released as sources of resistance to aflatoxin contamination, exhibited significant GCA for reduced aflatoxin accumulation (Williams et al., 2002). Mp496, which was developed and released as a source of resistance to southwestern corn borer leaf feeding (Scott and Davis, 1981), also exhibited significant GCA for reduced aflatoxin accumulation (Williams et al., 2003).

This investigation was undertaken to compare aflatoxin accumulation in crosses among 10 maize germplasm lines with varying levels of resistance. In this investigation, unlike those previously reported, reciprocal crosses were evaluated and included in the diallel analysis. The second objective was to determine the importance of GCA, specific combining ability (SCA), and reciprocal effects on the inheritance of resistance to aflatoxin accumulation.

MATERIALS AND METHODS

A diallel cross of maize was produced from 10 inbred lines: CI66, GA209, NC408, Mo18W, Mp313E, Mp494, Mp715, Mp717, SC212m, and T173. GA209, SC212m, T173, and CI66 are highly susceptible to aflatoxin contamination, while Mo18W and NC408 have exhibited moderate resistance to aflatoxin contamination (Gardner et al., 2007; Williams, 2006). Mp313E, Mp715, and Mp717 were developed and released as sources of resistance to *A. flavus* infection and aflatoxin accumulation (Scott and Zummo, 1990b; Williams and Windham, 2001, 2006). Although Mp494 was also developed as a source of resistance to aflatoxin contamination, it has not been released. The 10 parental lines were crossed in all combinations to produce 90 reciprocal F_1 hybrids.

The resulting hybrids were planted on 24 Apr. 2005 and 14 Apr. 2006 in a Leeper silty clay loam (fine, smectitic, nonacid, thermic Vertic Epiaquepts) soil at Mississippi State, MS. Hybrids were assigned to single-row plots that were 4 m long, spaced 0.97 m apart, and arranged in a randomized complete block design with four replications. Standard production practices were followed.

Seven days after silks had emerged from 50% of the plants in a plot, the top ear of each plant was inoculated with *A. flavus* isolate NRRL 3357, which is known to produce aflatoxin in maize, using the side-needle technique (Zummo and Scott, 1989). Using a tree-marking gun, a 3.4-mL suspension containing 3×10^8 *A. flavus* conidia was injected underneath the husks into the side of the ear. Inoculum was prepared as described by Windham and Williams (2002).

Ears were harvested approximately 60 d after inoculation and dried at 38° for 7 d. The ears from each plot were bulked and shelled. The grain was thoroughly mixed and ground using a Romer mill (Union, MO). The concentration of aflatoxin in a 50-g sample was determined using Vicam's Afla Test (Watertown, MA), a procedure that detects aflatoxin at levels as low as 1 ng g^{-1}.

Aflatoxin values were transformed as $\ln(\gamma + 1)$, where γ is the concentration of aflatoxin in a sample, before statistical analysis. The transformation was performed to provide a more normally distributed data set. The data were analyzed using the SAS General Linear Models procedure (SAS Institute, 2003). Variance was partitioned, using DIALLEL-SAS (Zhang and Kang, 1997, 2003) based on Griffing's (1956) Method 3, Model I, into GCA, SCA, reciprocal, maternal, and nonmaternal components and their interactions with years. To test mean squares for GCA, SCA, reciprocal, maternal, and nonmaternal components for significance in the two-year analysis, the interaction between years and the corresponding component was used as the error term for *F* tests. Estimates of GCA and SCA effects were calculated and their significance determined by *t* tests.

RESULTS AND DISCUSSION

The analysis of variance indicated significant differences among hybrids in both 2005 and 2006 (Table 1). Both GCA

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and SCA were significant sources of variation in both years and in the combined analysis. Differences among reciprocal crosses were highly significant in 2006 but were not significant in 2005 or in the two-year analysis. The interaction of reciprocal crosses and years was significant.

Because reciprocal effects were not significant in the combined analysis (Table 1), means for reciprocal hybrids were pooled for calculating hybrid means (Table 2). Levels of aflatoxin accumulation for the 45 hybrids ranged from 18 to 865 ng g^{-1} 2005 and 2006. Aflatoxin accumulation was lowest in Mp717 × Mp494, a cross between two germplasm lines developed as sources of resistance. Mp715 × NC408, Mo18W × Mp717, Mp 717 × NC408, Mp313E × Mp717, and Mp494 × NC408 also exhibited relatively low levels of aflatoxin accumulation that did not differ significantly from that of Mp717 × Mp494. Interestingly, the crosses between NC408 and the four germplasm lines selected for resistance were among those exhibiting the lowest levels of aflatoxin accumulation.

Estimates of GCA effects were highly significant for all parental lines in the two-year analysis (Table 3). The estimates for SC212m, T173, CI66, and GA209 were positive and highly significant in both 2005 and 2006 and in the twp-year analysis, indicating that these germplasm lines contribute to hybrids with increased susceptibility to aflatoxin contamination. Estimates of GCA effects for the other germplasm lines were highly significant but negative in the two-year analysis, indicating that these lines contribute to resistance to aflatoxin accumulation. In 2005 the GCA effect for Mo18W was negative but not significant. In 2006 GCA effects for Mp313E and Mp715 were negative but not statistically significant. The differences in GCA effects between years for Mo18W, Mp313E, and Mp715 undoubtedly contributed to the highly significant GCA × year interaction (Table 1).

General combining ability effects estimated from the combined analysis indicate that Mp717 made the greatest contribution to reduced aflatoxin contamination in the hybrids. Although NC408 was not developed as a source of resistance to aflatoxin contamination, the GCA effect for this line ranked second among those lines with negative estimates. Scott and Zummo (1990a) reported that SC76, one of the inbred lines from which NC408 was derived, exhibited moderate resistance to kernel infection by *A. flavus*.

Of the 45 hybrids, SCA effects were significant for only 10 (Table 2). Estimated effects for GA209 × Mp313E, CI66× Mp715, Mp715 × NC408, and Mp717 × Mp494 were significant and negative. Aflatoxin accumulation in these hybrids was lower than predicted by their general performance. Because the mean square for reciprocal crosses was not significant in the two-year analysis (Table 1), estimates of reciprocal effects were not calculated.

Years, hybrids × years, and GCA × years were highly significant sources of variation (Table 1). It is not uncommon for environmental effects and genotypes × environment interactions to contribute significantly to variation associated with aflatoxin accumulation in maize grain. Drought, high temperatures, and insect damage are among factors linked to highly variable responses to environment. The variability in responses of maize inbred lines and hybrids to environments provides a challenge those breeding for resistance to aflatoxin accumulation in maize: variation associated with environments is an obstacle to accurate determination of the genetic potential of inbred lines to reduce aflatoxin contamination in their hybrids.

The lack of significance for reciprocal, maternal, and nonmaternal effects in the multiyear analysis indicates that extra-nuclear factors are not important in the inheritance of resistance to aflatoxin accumulation in this set of crosses. Breeding strategies for developing aflatoxin-resistant maize germplasm should, therefore, focus on nuclear factors. Quantitative trait loci (QTLs) associated with resistance to aflatoxin accumulation in Mp313E have been identified in a population of Mp313E × B73 $F_{2:3}$ families (Brooks et al., 2005). Efforts to identify QTLs and ultimately specific genes associated with resistance to aflatoxin contamination in not only Mp313E but also Mp715 and Mp717 are currently underway.

The results of this investigation indicate that germplasm lines Mo18W, Mp313E, Mp715, Mp494, NC408, and Mp717 would be useful in developing aflatoxin-resistant

Table 1. Diallel analysis of variance for aflatoxin contamination of grain harvested from reciprocal single crosses of a diallel cross grown at Mississippi State, MS, in 2005 and 2006.

Source	df	Mean squares		
		2005	2006	Over years
		ln (y + 1)†		
Years	1	–	–	9.02**
Reps (yr)	6	–	–	1.29
Hybrids	89	5.21**	5.36**	8.56**
GCA‡	9	39.92**	28.17**	63.56**
SCA‡	35	1.92*	3.26**	3.57**
Reciprocals	45	0.83	2.45**	1.42
Maternal	9	0.66	1.35	0.69
Nonmaternal	36	0.88	2.72**	1.61
Hybrids × years	89	–	–	2.02**
GCA × years	9	–	–	4.43**
SCA × years	35	–	–	1.60
Reciprocals × years	45	–	–	1.85*
Maternal × years	9	–	–	1.32
Nonmaternal × years	36	–	–	1.99*
Error	267, 534§	1.18	1.05	1.12

*Significant at $P < 0.05$.

**Significant at $P < 0.01$.

†y = the concentration of aflatoxin in a sample.

‡GCA, general combining ability; SCA, specific combining ability.

§df = 267 for individual years and 534 for combined years.

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Table 2. Mean aflatoxin accumulation and estimates of specific combining ability (SCA) effects for F_1 hybrids comprising a diallel cross grown at Mississippi State, MS, in 2005 and 2006.

Hybrid	Aflatoxin† Geometric mean‡	Aflatoxin† Logarithmic mean	SCA effects
	ng g^{-1}	ln (y + 1)§ ng g^{-1}	
Cl66 × SC212m	865	6.76	0.15
T173 × SC212m	786	6.67	–0.13
Cl66 × T173	707	6.56	0.18
T173 × GA209	524	6.26	–0.11
GA209 × SC212m	505	6.23	–0.38
SC212m × NC408	492	6.20	0.80**
Cl66 × GA209	439	6.09	–0.10
Mp715 × SC 212m	372	5.92	0.40
Mo18W × GA209	371	5.92	0.67**
Mp313E × SC212m	319	5.77	0.22
T173 × Mp715	308	5.73	0.44
Mp313E × T173	291	5.68	0.40
GA209 × Mp494	260	5.56	0.54*
Cl66 × Mp 717	234	5.46	0.80**
Mp715 × GA209	213	5.37	0.27
T173 × NC408	202	5.31	0.14
Mo18W × SC212m	196	5.28	–0.39
Mo18W × T173	185	5.23	–0.21
Mo18W × Cl66	179	5.19	–0.06
Cl66 × NC408	178	5.18	0.21
Mp494 × SC212m	149	5.01	–0.44
GA209 × NC408	148	5.01	–0.03
Mp313E × Cl66	145	4.98	–0.14
T173 × MP494	140	4.95	–0.27
Cl66 × Mp494	131	4.88	–0.15
Mp717 × SC212m	125	4.84	–0.24
Mp313E × Mp494	112	4.73	0.76**
GA209 × Mp717	93	4.54	–0.11
Mp715 × Mp717	85	4.45	0.88**
TI73 × Mp717	83	4.43	–0.41
Mo18W × Mp313E	81	4.41	0.22
Mo18W × Mp494	79	4.38	0.29
GA209 × Mp313E	72	4.29	–0.83**
Cl66 × Mp715	66	4.20	–0.90**
Mo18W × NC408	55	4.03	–0.01
Mo18W × Mp715	54	4.00	–0.16
Mp715 × Mp494	54	4.00	0.07
Mp313E × Mp715	41	3.74	–0.31
Mp313E × NC408	39	3.68	–0.23
Mp494 × NC408	35	3.58	–0.23
Mp313E × Mp717	34	3.57	–0.03
Mp717 × NC408	30	3.44	–0.01
Mo18W × Mp717	29	3.39	–0.33
Mp715 × NC408	24	3.20	–0.69**
Mp717 × Mp494	18	2.94	–0.56*
LSD (0.05)		0.73	

*Significantly different from 0 at $P < 0.05$.

**Significantly different from 0 at $P < 0.01$.

†Data were transformed [ln(y + 1)] before analysis.

‡Geometric mean was calculated by converting logarithmic mean to original scale after analysis.

§y = the concentration of aflatoxin in a sample.

Table 3. Estimates of general combining ability (GCA) effects for 10 inbred parental lines of a diallel cross of maize grown at Mississippi State, MS in 2005 and 2006.

Parental inbred	GCA effect 2005	GCA effect 2006	GCA effect Over years
	ln (y + 1)† ng g^{-1}		
SC212m	1.04**	1.08**	1.06**
T173	0.89**	0.76**	0.83**
Cl66	0.75**	0.52**	0.64**
GA209	0.76**	0.51**	0.63**
Mo18W	–0.08	–0.52**	–0.29**
Mp313E	–0.64**	–0.20	–0.42**
Mp715	–0.66**	–0.23	–0.44**
Mp494	–0.62**	–0.42**	–0.52**
NC408	–0.31**	–0.84**	–0.57**
Mp717	–1.13**	–0.66**	–0.89**

**Significantly different from 0 at $P < 0.01$.

†y = the concentration of aflatoxin in a sample.

maize germplasm lines or hybrids. Breeding strategies that maximize the use of GCA should be effective. Although conventional breeding methods based on phenotypic selection should produce maize germplasm with resistance to aflatoxin contamination, all lines exhibiting significant negative GCA effects should be useful as sources of QTLs for resistance. Identification of QTLs that could be used in marker-assisted selection would facilitate the transfer of resistance to aflatoxin contamination from these germplasm lines into commercially available maize hybrids.

Acknowledgments

The authors express their appreciations to M.N. Alpe, G.A. Matthews, and L.T. Owens for technical assistance and to S.S. Pitts for assistance in preparing the manuscript. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by USDA.

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